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Molecular cloning, expression and radiation hybrid mapping of the bovine deiodinase type II (*DIO2*) and deiodinase type III (*DIO3*) genes[1]

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Summary

Thyroid hormones play a critical role in mammalian development and metabolism. Their activity is regulated in a complex, tissue-specific manner by three isoforms of deiodinases. The goal of this study was to sequence the full-length bovine type II deiodinase (*DIO2*) and type III deiodinase (*DIO3*) cDNAs and characterize mRNA expression levels of each of the three deiodinase isoforms in several bovine tissues. Sequencing of bovine *DIO2* and *DIO3* cDNAs revealed a high degree of predicted amino acid sequence identity with their orthologs in other mammalian species and demonstrated the conservation of selenocysteine residues within the catalytic domains of both bovine proteins. Bovine *DIO2* and *DIO3* were positioned on chromosomes 10 and 21, respectively, by radiation hybrid mapping. Expression patterns of the three deiodinase isoforms were similar for deiodinase type I (*DIO1*) and *DIO2* to those observed in other species. Expression level of *DIO3* transcripts was greatest in mammary gland and kidney, although low-level expression was detected in most tissues sampled. Results of this work will aid in the study of deiodinase gene expression and thyroid hormone regulation in cattle.

Keywords deiodinase, gene expression, gene mapping, thyroid hormone metabolism.

Thyroid hormones are critical for normal cellular differentiation, fetal development, linear growth in juveniles, and metabolism and reproduction in adults (Norris 1997). Thyroid hormone activity is regulated in a tissue-specific manner by three deiodinases, types I, II and III. Type I deiodinase (DIO1), found primarily in the liver and kidney, catalyzes 5′-deiodination of the prohormone thyroxine (T4) into its active form, triiodothyronine (T3) and is a principal regulator of circulating concentrations of T3. Likewise, type II deiodinase (DIO2) catalyzes the conversion of T4 to T3, but at the local tissue level where thyroid hormone activity must be tightly regulated. Metabolism of T3, in turn, is controlled by type III deiodinase (DIO3), which converts T3 into a biologically inactive form. Expression of DIO3 is greatest during pregnancy in placenta, uterus and fetal tissues and appears to function in the protection of developing embryonic tissues from excessive T3 levels (St Germain 1994).

Deiodinases are seleoenzymes that require selenocysteine (SeCys) residues within the catalytic domain for functional activity. The SeCys residues are encoded by the *opal* stop codon, UGA, but are differentiated by the presence of a SeCys insertion sequence element within the 3′-untranslated region (3′-UTR) of the transcript. Nucleotide sequences of the three deiodinase isoforms have been characterized in a number of species including humans, rodents, fish, and chicken and exhibit a high degree of sequence identity within the catalytic domain across species, as well as within each deiodinase isoform.

To date, the pig is the only domestic livestock species in which deiodinase mRNAs have been fully characterized, although a partial bovine *DIO1* sequence is available in GenBank (AF318504) and the gene was previously mapped to bovine chromosome 3 (Connor *et al.* 2003). The objectives of this study were to determine the full coding sequences of bovine *DIO2* and *DIO3* mRNAs, map the *DIO2* and *DIO3* genes on the bovine genome, and survey mRNA expression levels of the three deiodinase isoforms in bovine tissues.

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[1]The nucleotide sequence data reported in this paper were submitted to GenBank and assigned the accession numbers AY858551–AY858554. Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and in no way implies recommendation or endorsement by the US Department of Agriculture.

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Table 1 Primers used for RT-PCR, qRT-PCR, 5′-RACE and radiation hybrid mapping of the bovine deiodinases.

Primer name	Primer sequence (5′ → 3′)	Orientation	Temperature (°C)	Target	Method
DIO1-F	TGG TGG TAG ACA CAA TGA CGA A	Sense	52.0	DIO1	qRT-PCR
DIO1-R	GGC CAG ATT TAC CCT TGT AGG A	Antisense	52.0	DIO1	qRT-PCR
DIO2-1	TTC CAG TGT GGT GCA TGT CT	Sense	50.0	DIO2, putative exon 2	RT-PCR
DIO2-2	GCA CAT CGA TCT TCC TGG TT	Antisense	50.0	DIO2, putative exon 2	RT-PCR
DIO2-5′-RACE	GAA GGT GGC ATT CAG TTC CAT CCA CTG T	Antisense	68.0	DIO2, 5′-end	5′-RACE
DIO2-5′-UTR1	ACA AGG GAA CTG ACC CAG GA	Sense	55.0	DIO2, 5′-UTR	RT-PCR
DIO2-5′-UTR2	GGA AGT CAG CCA CGG ATG AG	Antisense	55.0	DIO2, 5′-UTR	RT-PCR
DIO2-3′-UTR1	CCA CCT TCT GGA CTT TGC CA	Sense	55.0	DIO2, 3′-UTR	RT-PCR
DIO2-3′-UTR2	CCA ATA GGG CTC TGT TGA AA	Antisense	55.0	DIO2, 3′-UTR	RT-PCR
DIO2-RH1	GCT GAC CTC AGA AGG AAT GC	Sense	55.0	DIO2, putative intron 1	RH mapping
DIO2-RH2	GGC ATT CAG TTC CAT CCA CT	Antisense	55.0	DIO2, putative intron 1	RH mapping
DIO2-RH3	CAT GAA GCA CGC ACT CAC TT	Sense	55.0	DIO2, putative intron 1	RH mapping
DIO2-RH4	CAG AAA GAG GTG CCA TGT CC	Antisense	55.0	DIO2, putative intron 1	RH mapping
DIO2-F	CCA CCT TCT GGA CTT TGC CA	Sense	54.0	DIO2	qRT-PCR
DIO2-R	GGA AGT CAG CCA CGG ATG AG	Antisense	54.0	DIO2	qRT-PCR
DIO3-1	GGG AAG GTA GGG GGA CTT T	Sense	55.0	DIO3, full-coding region	RT-PCR
DIO3-2	TGT TTG CAC GTG GGC TTC	Antisense	55.0	DIO3, full-coding region	RT-PCR
DIO3-RH1	GGG AAG GTA GGG GGA CTT T	Sense	57.0	DIO3	RH mapping
DIO3-RH2	GGC AGG ACC ACC TCA GAG T	Antisense	57.0	DIO3	RH mapping
DIO3-F	TCA CTC CCT GAG GCT CTG	Sense	61.0	DIO3	qRT-PCR
DIO3-R	CCC AGT AAA TGC TTA CGG ATG	Antisense	61.0	DIO3	qRT-PCR
ATP5B-F	CCC AGT AAA TGC TTA CGG ATG	Sense	58.2	ATP5B	qRT-PCR
ATP5B-R	AAT GGT CCT TAC TGT GCT CTC	Antisense	58.2	ATP5B	qRT-PCR

The full-length coding sequence of *DIO2* (accession no. AY858551) was obtained by RT-PCR and 5′-rapid amplification of cDNA ends (5′-RACE) using total RNA isolated from mammary epithelium as template. Table 1 summarizes primer sequences used for all amplification reactions. Initial RT-PCR reactions were performed using primers DIO2-1 and DIO2-2 and the ProSTAR HF Single-Tube RT-PCR System (Stratagene, La Jolla, CA, USA) according to manufacturer's instructions. Primer sequences were based on human mRNA sequence (NM_001007023) and positioned within regions highly conserved across species. The resulting 330-bp amplification product was cloned into a plasmid vector and sequenced in both directions. All sequencing was performed using a CEQ8000 automated sequencer and DTCS Quickstart chemistry (Beckman Coulter, Fullerton, CA, USA). The 5′-RACE was conducted using the SMART RACE cDNA Amplification Kit (Clontech, Palo Alto, CA, USA) according to kit instructions to obtain 152 bp of additional 5′-cDNA sequence. The complete 5′- and 3′-coding regions were obtained by RT-PCR using primers DIO2-5′-UTR1 with DIO2-5′-UTR2 (499 bp), and DIO2-3′-UTR1 with DIO2-3′-UTR2 (*c.* 577 bp) respectively. Primer sequences were based on bovine sequences obtained from previous reactions and highly conserved 5′- and 3′-UTR sequences of human, porcine (NM_001001626) and murine (NM_010050) *DIO2*. All products were cloned into a plasmid vector and sequenced.

The bovine *DIO2* cDNA encodes a predicted 269-amino acid protein and shares 95, 92 and 91% identity with porcine, human and murine DIO2 amino acid sequences respectively. The presence of two putative SeCys residues was detected at positions 133 and 266 of the predicted protein sequence, consistent with the human and murine proteins (See Supplementary material).

For sequencing of full-length bovine *DIO3* mRNA, primer sequences DIO3-1 and DIO3-2 targeting 5′- and 3′-UTR were selected based upon sequence of a partial bovine EST (AW481205) and ovine *DIO3* mRNA (AY656759). PCR amplification was conducted using iQ Supermix (Bio-Rad Laboratories, Hercules, CA, USA) and the resulting 941-bp product was cloned into a plasmid vector and sequenced. The bovine *DIO3* transcript encodes a predicted 278-amino acid protein sharing 96, 95 and 91% identity with porcine, human and mouse proteins, respectively, and exhibits a conserved SeCys residue at amino acid position 144 (See Supplementary material). As observed in human and rat (St Germain & Galton 1997; Bianco *et al.* 2002), an alignment of bovine DIO2 and DIO3 amino acid sequences revealed conservation of SeCys residues within active centres of both enzymes and conservation of histidine residues (equivalent to His165 and His185 in human DIO2), critical for proper enzyme structure and substrate affinity (Bianco *et al.* 2002). These data further support the high degree of conservation of deiodinase isoforms across species.

For physical mapping of *DIO2* and *DIO3*, DNA from the Roslin-Cambridge 3000-rad bovine/hamster radiation hybrid (RH) panel (Invitrogen Corp., Carlsbad, CA, USA) was screened as previously described (Connor *et al.* 2004) using bovine-specific PCR primers for each gene (Table 1). Primers for *DIO2* resulted in amplification of an *c.* 8-kb product that was end-sequenced for verification of gene identity. Partial sequences were submitted to the GenBank STS database (AY858553 and AY858554). To facilitate screening of the RH panel by PCR, nested primers (DIO2-RH3 and DIO2-RH4) were designed from AY858554 to target amplification of a smaller, 390-bp product. For mapping of *DIO3*, primers (DIO3-RH1 and DIO3-RH2) were designed based on bovine sequence AW481205 to amplify a 385-bp product. Sequencing confirmed amplification of targeted regions. Chromosomal positions were identified by two-point linkage analysis against 1200 markers published for the 3000-rad panel (Williams *et al.* 2002). *DIO2* was mapped to bovine chromosome 10 (BTA10), near microsatellite marker BMS614 (LOD = 18.65; distance = 0.059 cR). *DIO3* was positioned near microsatellite marker BMS2382 on BTA21 (LOD = 9.04; distance = 0.355 cR). In human, the *DIO2* and *DIO3* genes map to chromosome 14q24.2 and 14q32 respectively. Mapping of *DIO2* and *DIO3* in cattle provides further evidence of the conserved synteny between human chromosome 14 with BTA10 and 21.

To survey expression of *DIO1*, *DIO2* and *DIO3* mRNAs, total RNA was isolated from heart, liver, mammary gland, thyroid gland, diaphragm, kidney, hypothalamus, pituitary and lung obtained from cattle at slaughter. Thyroid samples were from 16-month-old steers and pituitary samples were from steers at *c.* 370-kg body weight. Remaining tissues were from 8-month-old Holstein heifers. Two animals were represented for each tissue (except kidney where only one sample was of suitable quality for analysis). The RNA quantity and quality were determined using a 2100 Bioanalyzer (Agilent Technologies, Foster City, CA, USA). First strand cDNA was synthesized from 500 ng of total RNA from each sample using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories). Negative controls (minus reverse transcriptase) were also generated for each RNA sample. Subsequent PCR was conducted using iQ SYBR Supermix (Bio-Rad Laboratories) in a 25-μl reaction volume according to manufacturer's instructions.

Abundance of deiodinase transcripts and the housekeeping gene, ATP synthase (*ATP5B*) were determined by absolute quantitative real-time RT-PCR using the iCycler iQ Real-time PCR Detection System (Bio-Rad Laboratories). Primer sequences and annealing temperatures are presented in Table 1. Identity of amplification products was confirmed by direct sequencing. Cycling conditions were 95 °C for 3 min followed by 45 cycles of 94 °C for 15 s, annealing temperature for 30 s and 72 °C for 30 s. Standard curves ranging from 10 to 2×10^6 molecules were generated for each gene using purified double-stranded DNA containing the amplification region of interest and analyzed in duplicate. A negative control containing water as template was included with each assay. Amplification efficiencies were 98.2, 89.3, 96.3 and 88.1% for *DIO1*, *DIO2*, *DIO3* and *ATP5B* respectively. Correlation coefficients for each standard curve were ≥0.997. Transcript abundance in all negative controls was below the detection limit. Quantities of deiodinase transcripts expressed in each tissue are presented in Table 2.

Amplification of *ATP5B* from each tissue confirmed RNA sample integrity was suitable for RT-PCR. Transcripts of *DIO1* were above the limit of assay detection in liver samples only, although products were obtained after 45 cycles of amplification from thyroid, kidney and mammary gland, suggesting expression at very low levels in these tissues. In support of this finding, a partial bovine sequence for *DIO1* mRNA (AF318504) was cloned from thyroidal tissue and DIO1 enzyme activity was demonstrated previously in bovine thyroid gland (Connor *et al.* 2001). Our results are in agreement with reported enzyme activities in bovine tissues (Kahl *et al.* 1998) and expression patterns observed in other mammalian species (Beech *et al.* 1993).

Table 2 Abundance of deiodinase type I (*DIO1*), type II (*DIO2*), type III (*DIO3*), and the house-keeping gene, *ATP5B* mRNA transcripts in various bovine tissues as determined by real-time quantitative RT-PCR.

	Molecules/μg total RNA (mean ± SE)			
Tissue	DIO1	DIO2	DIO3	ATP5B
Diaphragm	ND	8260 (±3900)	2650 (±150)	6.1×10^6 ($\pm 6.3 \times 10^5$)
Kidney	ND	518	10 200	7.2×10^6
Heart	ND	1824 (±456)	1287 (±910)	2.1×10^7 ($\pm 8.9 \times 10^6$)
Hypothalamus	ND	42 660 (±37 740)	2280 (±1280)	9.2×10^5 ($\pm 7.9 \times 10^5$)
Liver	960 (±274)	ND	ND	
Lung	ND	160 (±90)	4150 (±3150)	
Mammary gland	ND	174 300 (±41 700)	139 800 (±12 200)	3.9×10^6 ($\pm 8.2 \times 10^5$)
Pituitary gland	ND	212 000 (±132 000)	2509 (±671)	7.2×10^5 ($\pm 3.3 \times 10^5$)
Thyroid gland	ND	291 700 (±210 300)	3701 (±1939)	7.1×10^5 ($\pm 4.3 \times 10^5$)

ND, below assay detection limit.

Transcripts of *DIO2* were most abundant in thyroid, mammary and pituitary glands, followed by hypothalamus. Low levels were also detected in diaphragm, heart, kidney and lung. It appears that expression patterns of *DIO2* in cattle are similar to those reported for humans (Salvatore *et al.* 1996, http://genecards.weizmann.ac.il). Expression of *DIO3* transcripts was greatest in mammary gland and kidney and low in most remaining tissues sampled. Based on our results and a survey of *DIO3* expression from human tissues (http://genecards.weizmann.ac.il/), it appears that most adult tissues express *DIO3* mRNA at low levels.

Overall, the amino acid sequences of bovine deiodinases type II and III are highly conserved with other mammalian species and expression patterns of the three isoforms appear to be similar to those observed in other species. From our results, it appears that the mammary gland is a primary source of *DIO2* and *DIO3* mRNAs and previous research suggests that deiodinases may play a role in regulating local metabolic activity of the mammary gland during lactation (Kahl *et al.* 1998; Capuco *et al.* 1999). Studies to examine the role of DIO2 and DIO3 during lactation in cattle are currently underway within our laboratory.

Supplementary material

The following material is available from: http://www.blackwellpublishing.com/products/journals/suppmat/AGE/AGE1282/AGE1282sm.htm

Appendix S1 Predicted amino acid sequence alignment of bovine, porcine, human and mouse *DIO2* and *DIO3*.

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